Tissue transglutaminase in fibrosis - more than an ECM crosslinker

Benn, Mario C ; Weber, Willi ; Klotzsch, Enrico ; Vogel, Viola ; Pot, Simon A

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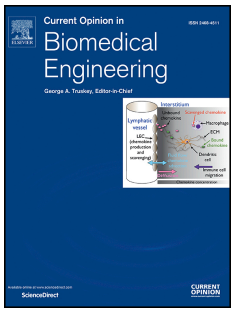

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Tissue transglutaminase in fibrosis - more than an ECM crosslinker

Mario C. Benn[#,1], Willi Weber[@], Enrico Klotzsch[#,@], Viola Vogel[#], Simon A. Pot[#,*,1]

[#] Laboratory of Applied Mechanobiology, Department for Health Sciences and Technology, ETH Zürich, 8093 Zürich, Switzerland
[@] Institute of Biology, Experimental Biophysics/ Mechanobiology, Humboldt Universität zu Berlin, 10115 Berlin, Germany
* Ophthalmology Unit, Equine Department, Vetsuisse Faculty, University of Zurich, 8057 Zurich, Switzerland

[1] equal contribution

ORCID IDs:
Mario C. Benn: 0000-0002-5345-4554
Willi Weber: 0000-0003-4110-6744
Enrico Klotzsch: 0000-0002-7577-9042
Viola Vogel: 0000-0003-2898-7671
Simon A. Pot: 0000-0002-8782-7144

Corresponding Author:
Prof. Simon A. Pot
Ophthalmology Unit, Equine Department
Vetsuisse Faculty, University of Zurich
Winterthurerstrasse 260
CH-8057 Zürich, Switzerland
Tel.: +41-44-635-9030
Fax: +41-44-635-8940
Email: spot@vetclinics.uzh.ch

Highlights of the review:

- **TG2 has critical and multifaceted roles beyond ECM crosslinking**
- **TG2 function depends on its localization and allosterically-regulated conformation**
- **TG2 is upregulated in the pathogenesis of a wide variety of chronic diseases**
- **Many similarities exist between the roles of TG2 in the scar tissue and tumor stroma**
- **TG2 facilitates both TGF-β1 storage in the ECM and TGF-β1 activation**
- **TG2 is an important cell adhesion and signaling protein at the cell membrane**

Keywords:

tissue transglutaminase, TG2, tTG, cross-linking, non-enzymatic, fibrosis, ECM, TGF-β1

Abstract:

Tissue transglutaminase (TG2) is upregulated in the pathogenesis of a wide variety of chronic diseases. In this review special emphasis will be placed on fundamental mechanisms underlying the critical role of TG2 in fibroproliferative disorders. TG2 is best known for its cross-linking capacities in the extracellular space but has many critical and multifaceted roles beyond protein cross-linking, which are driven by the conformation and specific localization of the molecule.

As extracellular crosslinker TG2 promotes fibrotic disease through the storage of latent TGF-β1 in a stiffened extracellular matrix (ECM). As membrane-bound cell adhesion cofactor and signaling protein and intracellular crosslinker or G-protein, TG2 promotes fibrotic disease through cell survival and profibrotic pathway activation on a signaling, transcriptional and translational level. Similarities between the roles that TG2 plays in scar tissue and in the tumor stroma suggest that a deeper understanding of key common pathways in disease pathogenesis and progression might lead to the identification of novel treatment targets and the development of new drugs and diagnostic methods.

TG2 - more than an ECM crosslinker

Tissue transglutaminase (TG2), also named tTG, EC 2.3.2.13, is the most studied representative of a structurally and functionally related family of proteins of which nine members have been identified in humans [1,2]. TG2 is best known for its catalytic transamidation activity, resulting in the Ca^{2+} dependent post-translational formation of covalent isopeptide bonds between glutamine and lysine residues [1]. Beyond its catalytic core, TG2 consists of an N-terminal β-sandwich and two C-terminal β-barrel domains (Figure 1, left) [3–5]. Far less understood are TG2s multiple functions at the cell membrane, in the cytoplasm and in the cell nucleus, such as adhesion, migration, growth, proliferation, survival, apoptosis, differentiation and phenotype modulation [6,7]. TG2 is an integrin and syndecan-binding adhesion co-receptor for fibronectin (Fn). Approximately 5–40% of β1 integrins are in complex with TG2 and almost all cell-membrane bound TG2 forms 1:1 complexes with integrins [8,9], which suggests a prominent role for TG2 at the cell membrane. While the focus is often on its crosslinking capacities in the extracellular space, TG2 is also active through non-enzymatic protein–protein interactions in both the extra- and intracellular space as further highlighted below. The reader is also referred to the following comprehensive review [10].

TG2 is upregulated in the pathogenesis of a wide variety of chronic diseases

In most cases, the pathophysiological significance of TG2-induced modifications remains unclear. However, it is well documented that TG2 is involved in the pathogenesis of a wide variety of diseases, most notably neoplastic and fibroproliferative, including most malignant cancers and pulmonary, kidney and cardiac fibrosis [11–14]. TG2 expression is highly correlated with cancer cell survival, malignancy, metastasis and treatment resistance [13,15]. TG2 morphologically and functionally ‘shapes’ the tumor and scar tissue stroma through ECM cross-linking and binding of TGF-β1 to the ECM, thus priming TGF-β1, one of the most effective profibrotic stimuli, for release and activation [16,17]. Also, through its cell attachment and signaling mediator functions and its intracellular signaling and crosslinking functions, TG2 can promote tumor cell survival and the development of fibrotic (scar) cell phenotypes [7,8,18–23]. Major attempts are thus underway to develop potent TG2 inhibitors.

Environmental sensing through major conformational TG2 changes

The biochemical functions of TG2 largely depend on its molecular conformation (Figure 1). Various environmental factors cause allosteric conformational changes and include extracellular, intracellular and intranuclear Ca^{2+} concentrations, GDP and GTP concentrations and MMP-mediated release of membrane bound TG2 into the ECM [7,9]. When bound to GTP or GDP, TG2 adopts a closed conformation with the two C-terminal β-barrel domains folded in and blocking substrate access to the catalytic site. However, with excess Ca^{2+} levels, TG2s affinity for GDP and GTP is reduced leading to a very large change in molecular shape presenting an open molecular conformation with an accessible active catalytic cross-linking site (Figure 1b) [3,24]. Low Ca^{2+} and high GDP/GTP concentrations in the cytoplasm cause TG2 to adopt a predominantly closed conformation inside the cell [25]. The conformation that TG2 adopts at the cell membrane is largely unknown. No structure has yet been solved of TG2 complexed to any binding partner even though Fn can bind to the open and closed conformations of TG2 [5]. Closed state cytosolic TG2 can bind to heparan sulfate epitopes on

exosome membrane associated Syndecan-4 molecules which have a high binding affinity for the closed conformation of TG2. Syndecan-4-dependent translocation of TG2 from the cytoplasm to the extracellular space takes place by fusion of exosomes with the outer cell membrane [9,26–28]. This mechanism was identified in fibrotic kidney disease by Furini et al. [27]. Syndecan-4-associated TG2 delivered to the cell surface can interact with integrins and fibronectin to form membrane associated protein complexes exerting cell adhesion/receptor protein functions (Figure 2, 3) [7,29]. MMP2/9 activity can lead to Syndecan-TG2 shedding from the cell surface (Figure 3)[9]. Finally, TG2 predominantly adopts an open conformation in the extracellular space due to high Ca^{2+} and low GDP/GTP concentrations [24]. Formation of two disulfide bonds between cysteine residues under oxidizing conditions at the catalytic site renders TG2 catalytically inactive in its open conformation [30]. Active oxygen reduction mechanisms in the ECM therefore promote the catalytic ECM cross-linking activity of TG2 in the extracellular space [31]. Mechanical forces within the physiological range for cells have been shown to induce allosteric conformational and functional changes in proteins [32]. Also, TG2 is concentrated at focal adhesion sites [8,18] and the integrin and Fn-binding sites are located at opposite sides of the TG2 molecule with the catalytically active site and hinge region in between (Figure 1) [4]. It is therefore likely that cells that generate mechanical forces and pull at adhesion sites that are physically connected to the extracellular matrix can induce TG2 'opening' and catalyze disulfide bond reduction, thus inducing and stabilizing an open catalytically active conformation of TG2 [3,4].

Extra- and intracellular: Transamidation and cross-linking functions of TG2 / TGF-β1 storage in the ECM

The catalytic activity of TG2 can affect protein conformation by generating intramolecular cross-links and can catalyze the formation of covalently linked dimers, oligomers, and polymers [6]. The transamidation activity of TG2 results in protease-resistant inter- or intramolecular isopeptide bonds which effectively cross-link ECM fibrils, stiffening the ECM and protecting the ECM from proteolytic degradation [24]. A matrix reservoir of inactive TGF-β1 is formed via TG2-mediated cross-linking of latent TGF-β1 binding protein (LTBP-1) to the ECM (Figure 2, 3) [33,34]. ECM-bound TGF-β1 can be activated via mechanical release (Figure 3), which is especially effective when the LTBP-1 is bound to a stiff, deformation resistant ECM that counters cell-applied forces [17,33,35,36]. As such, TGF-β1 activation is thought to be facilitated by TG2 since TG2 cross-links LTBP to the ECM and can turn a compliant matrix into a stiffer matrix via cross-linking of various ECM components [37]. ECM prestress sensitizes latent TGF-β1 for activation [36], which might explain increased TGF-β1 release from stiffened fibrotic tissues. Since increased and dysregulated expression, deposition and cross-linking of ECM cause loss of vital organ function in fibrosis, TG2 and TGF-β1 are explored as targets for the treatment of fibrotic diseases.

Cell membrane: TG2 regulates cell adhesion and ligand affinity

TG2 binds to the fibronectin type I modules $FnI_{7–9}$ of the collagen/gelatin binding site on the Fn molecule and interacts with cell membrane β1, β3 and β5 integrins [8,38–40] and/or syndecan-4 (Figure 3) [9,28,29]. Syndecan-4 and integrin signaling is coordinated via direct complex

formation of Fn-TG2, β-integrins and syndecan-4, indirect integrin cytoplasmic tail activation via Syndecan-4 activated PKCα or downstream convergence of integrin and syndecan signaling at p190RhoGAP (Figure 3) [29,41]. TG2-Fn-complexes offer cells an alternative binding site on Fn in addition to the classical integrin-Fn binding sites (e.g. RGD loop, synergy site) and facilitate RGD independent cell adhesion [28,29,42,43].

By promoting cell adhesion to Fn via cell surface integrins and syndecan-4, TG2 likely stimulates Fn fibril assembly through cell contraction-mediated Fn stretching and unmasking of Fn self-assembly sites [38,44]. Indirectly, integrin clustering and binding of TG2 at focal adhesions might concentrate TG2 catalyzed cross-linking of the dense Fn matrix at these sites as suggested by Zemskov et al. [18].

Proteolytic degradation of surface TG2 can shift cell-ECM recognition: cells cannot bind to collagen, if TG2 is bound to certain collagen-binding integrins, such as integrin α1β1, likely due to steric hindrance [18]. MT1-MMP and/or MMP-2 degrade integrin-associated cell surface TG2 and restore α1β1 integrin-collagen interactions, while at the same time suppressing cell adhesion and migration on fibronectin. This shift in cell-ECM recognition via dynamic regulation of surface TG2 can affect adhesion and migration of cells on various ECM ligands [7,18].

The importance of TG2 for cell adhesion is underlined by the discovery that TG2 was 1 out of only 10 proteins that consistently appeared in seven integrin adhesome datasets analyzed by Horton et al. [45].

Cell membrane: TG2 as signaling protein

Almost all cell membrane-associated TG2 is bound to integrins [8], where TG2 dimers facilitate integrin clustering formation (Figure 3)[7,19]. Further ECM components (e.g. Fn) and cell membrane protein receptors that bind next to fibronectin's RGD-specific integrin binding site (e.g. Syndecan-4, PDGF, VEGF, FGF and EGF receptors) can be engaged by these TG2-integrin complexes to execute various cell adhesion (described above) and signaling functions (Figure 3) [7,9,21,29]. TG2-integrin clusters, TG2-Fn-integrin and TG2-Fn-integrin-syndecan-4 complexes are known to activate well reviewed integrin signaling cascades, resulting in RhoA-ROCK activation, actin filamentation and downstream release and nuclear translocation of MRTF-A and YAP/TAZ (Figure 3) [7,8,19,46,47]. Nuclear MRTF-A and YAP/TAZ both act as transcription cofactors that promote expression of profibrotic genes, including αSMA, Tenascin-C and CTGF, which can influence the tumor stroma and cancer development and promote fibrotic disease [17,48,49].

Inside the cell: Enzymatic and non-enzymatic TG2 activity

With low Ca^{2+} and high GDP/GTP concentrations in the cytoplasm, TG2 binds to and hydrolyzes GTP, assumes a closed conformation and acts as a G protein. For example, TG2 binds to c-Src and PI3-kinase, facilitating c-Src-dependent phosphorylation of PI3-kinase which promotes cell survival [22]. Due to fluctuations in Ca^{2+} levels, cytoplasmic open conformation cross-linking activity of TG2 can also be observed with various specific and unspecific target proteins. For example, TG2 drives the constitutive activation of NF-κB through its cross-linking activity, which leads to increased TGF-β1 expression (e.g. fibrotic diseases, cancer stroma), promotion of epithelial-mesenchymal transformation (increasing tumor malignancy) and cell survival [23,49]. Similarly, RhoA cross-linking and activation leads to increased stress fiber formation [50] and

certain translation regulatory proteins like YB-1 can be crosslinked in a state that drives increased αSMA protein translation [20]. TG2 also regulates mitochondrial function and can initiate mitochondrial-driven apoptosis by crosslinking mitochondrial proteins in its open, catalytically active state [24,51]. At the same time, TG2 cross-linking stabilizes the structure of dying cells, prevents leakage of proteolytic enzymes and protects the environment of the cell from further damage [52]. Last, high TG2 levels increase autophagy of the tumor suppressor p53, which can help tumor cells escape apoptosis [53].
Nuclear TG2 has been shown to regulate gene expression via post-translational modification of transcriptional factors and related proteins, including Sp1, hypoxia inducible factor (HIF) 1 and histones [54]. The G-protein function of cytosolic TG2 typically enhances cell survival [22], whereas the transamidation activity of TG2 can lead to cell death or survival or even fibrotic changes [20,23,49–51,55], depending on the target protein(s). Nuclear localization of TG2 is generally protective against cell death [56,57].

TG2 as major player in the development and maintenance of fibroproliferative diseases

Fibroproliferative disorders cause approximately 45% of the mortality in the developed world and appear in a wide spectrum from systemic to organ-specific fibrotic diseases. Besides increased mechanical tissue tension and the presence of certain ECM components like cellular fibronectin's ED-A domain, TGF-β1 is one of the most effective profibrotic stimuli that drive the transformation of fibroblasts into myofibroblasts [17]. Myofibroblasts are the key players within fibroproliferative disorders [17,58]. They incorporate an excess of collagen and other fibrous proteins into the ECM while expressing strong contractile alpha smooth muscle actin (αSMA) positive stress fibers which contract the fibrotic matrix into a stiff, dysfunctional scar [17,33,48,59]. Interestingly, TG2 and TGF-β1 reinforce each other in the progressive fibrotic and tumor stroma microenvironment [49]. As described in previous sections, TG2 facilitates the activation of TGF-β1 in the ECM [16], the activation of profibrotic signaling cascades downstream from TG2-β-integrin-containing membrane signaling complexes [7] and the translation of αSMA [20]. TGF-β1 on the other hand promotes TG2 transcription [60,61] leading to self-amplification of TG2 and TGF-β1 in fibrotic tissues and explaining why both are upregulated in fibroproliferative disorders. The fact that TG2 knockout mice were protected against fibrosis [62,63] and that TG2 inhibition in mouse models of fibrosis significantly reduced the fibrotic phenotype [11,12,64] further underlines the critical role that TG2 plays in the pathogenesis and maintenance of fibrosis [65]. The relative contributions of catalytic and non-catalytic TG2 functions to healthy and diseased microenvironments still remain unknown. Inhibition of TG2s catalytic function can reduce cardiac fibrosis in vivo [11,64,66]. Wang et al. and Shinde et al. suggested further reduction of fibrosis via non-catalytic TG2-mediated mechanisms: reduced externalization of TG2 as a result of decreased interaction of intracellular TG2 with exosome membrane associated syndecan-4, decreased TGF-β1-induced myofibroblast formation and reduced activation of fibrosis-associated genes [11,64].

Outlook: TG2 as common denominator in fibroproliferative and neoplastic diseases

The consensus in cancer research seems to mirror our descriptions of the profibrotic functions of TG2 outlined above. Extracellular TG2 in its open enzymatically active conformation promotes cancer cell malignancy through crosslinking of the tumor stroma [49,55]. Intracellular

closed conformation TG2 promotes cancer cell survival and malignancy through survival pathway and EMT activation [22,49]. Stabilizing the open state of TG2 inside the cell promotes cell death and inhibits the malignant phenotype of cancer cells, thus representing a promising new avenue in the treatment of cancer [24,25]. Similarities between scar tissue and tumor stroma [67–69] suggest that the identification of key common pathways involved in the disease pathogenesis and progression might lead to the identification of novel treatment targets and the development of new drugs and diagnostic methods.

Beyond its role as ECM crosslinker, much future research is needed to understand the many roles of TG2, including cell adhesion stabilization and its nuclear functions. Also, translational research is required as just a few TG2 inhibitors are in clinical trials and none is available for clinical use [55].

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Declarations of interest: none

Figures + legends

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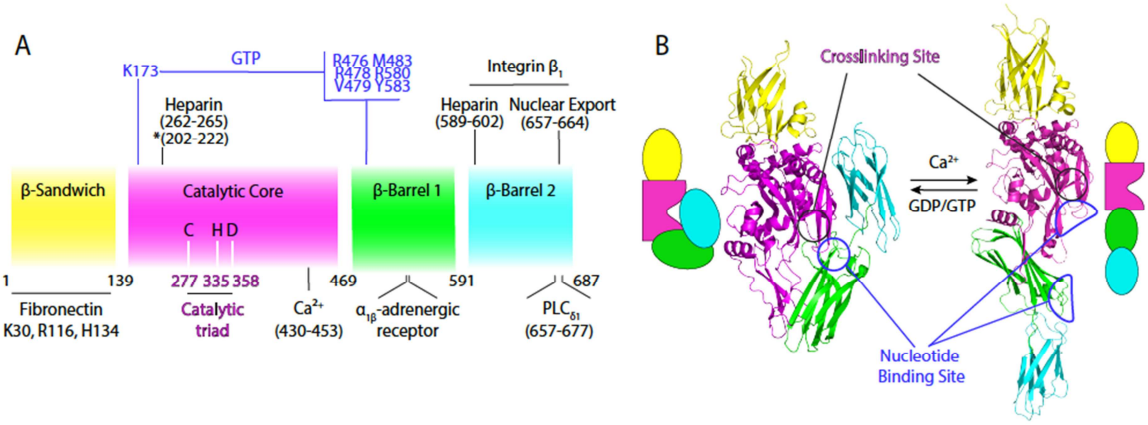

Figure 1:

Functional domains, binding sites and structure of tissue transglutaminase (TG2).

Beyond its catalytic core (140 - 454), TG2 consists of an N-terminal β-sandwich motif (1 - 139) and two C-terminal β-barrel domains(1: 469-591; 2: 592-687) [3–5]. The most relevant functional binding sites will be described briefly.

A) Recent studies identified residues K30, R116 and H134 on the N-terminal β-sandwich (yellow) as critical binding partners for the 42 kDa gelatin binding domain of Fn [5]. According to Belkin et al. TG2-integrin binding is disrupted upon deletion of C-terminal β-barrel 2 (blue), which indicates that integrin binding sites reside on that domain [4]. The catalytic core region (magenta) contains the active cross-linking site with the catalytic triad residues (C277, H335, D358). GTP (nucleotide) binding takes place on the catalytic core (K173) and β-barrel 1 (green) (R476k R478, V479, M483,R580, Y583) [70]. Heparin binding sites were identified on the catalytic core and β-barrel 2 [71]. (*) Wang et al. identified an alternative heparin binding site [9]. Ca^{2+}-binding to five of six Ca^{2+} binding sites on the catalytic core fosters allosteric catalytic site activation [72]. For information regarding further binding sites (α1β-adrenergic receptor, PLCδ1, nuclear export signaling peptide) the reader is referred to the following references [70,71].

B) X-ray crystallography demonstrated that enzymatic cross-linking activity of TG2 is only possible in the Ca^{2+}-induced open-state conformation (PDB: 2Q3Z, [3]) with exposed catalytic triad residues. Nucleotide binding induces the closed-state conformation (PBD: 1KV3) with catalytic triad residues blocked by β-barrels 1 and 2 [73]. However, since TG2 has many non-enzymatic functions, “conformational state” and “activity” must be carefully distinguished [24].

Figures are adapted and modified with permission from A [70] and B [24].

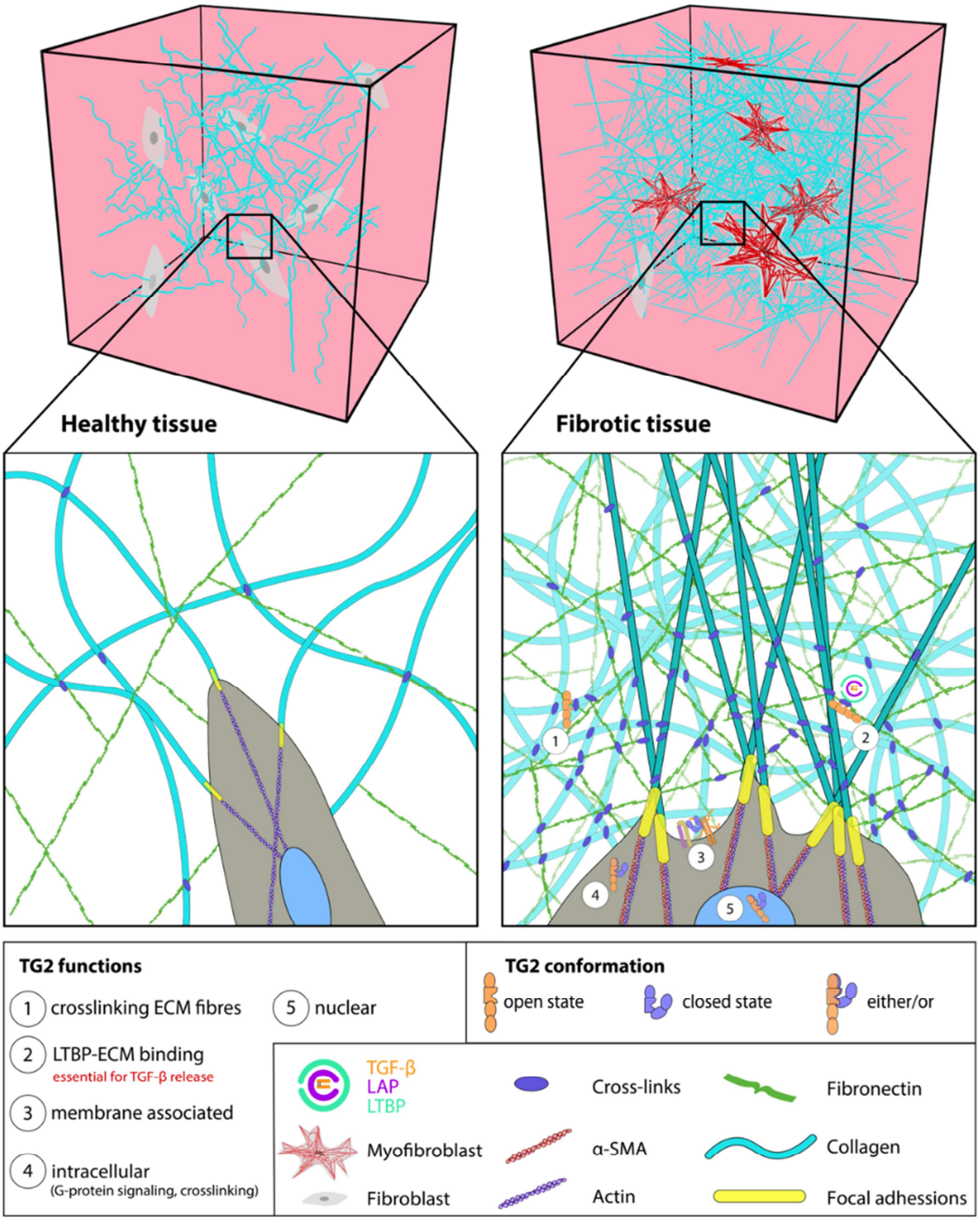

Figure 2:
TG2 functions depend on its localization, as well as its allosterically-regulated conformation as upregulated in many diseases.
Resident fibroblasts deposit and remodel the ECM in healthy tissues (top and middle left) [44], whereas αSMA expressing contractile myofibroblasts are the key players in fibrotic disease (top and middle right). Myofibroblast-dominated tissues are characterized by increased ECM fiber density and cross-linking. TG2 and TGF-β1 are both upregulated in fibrotic tissues. The open

conformation of TG2 cross-links ECM fibrils ① [1], and binds latent TGF-β1 via LTBP-1 to the ECM ② [1,34,74]. TG2 interacts in closed or unknown conformation with cell membrane proteins ③, resulting in downstream signaling effects [9,29,41]. Cytosolic ④ and nuclear ⑤ TG2 activate intracellular signaling pathways and downstream gene expression through enzymatic (open state TG2) and non-enzymatic (closed state TG2) mechanisms.

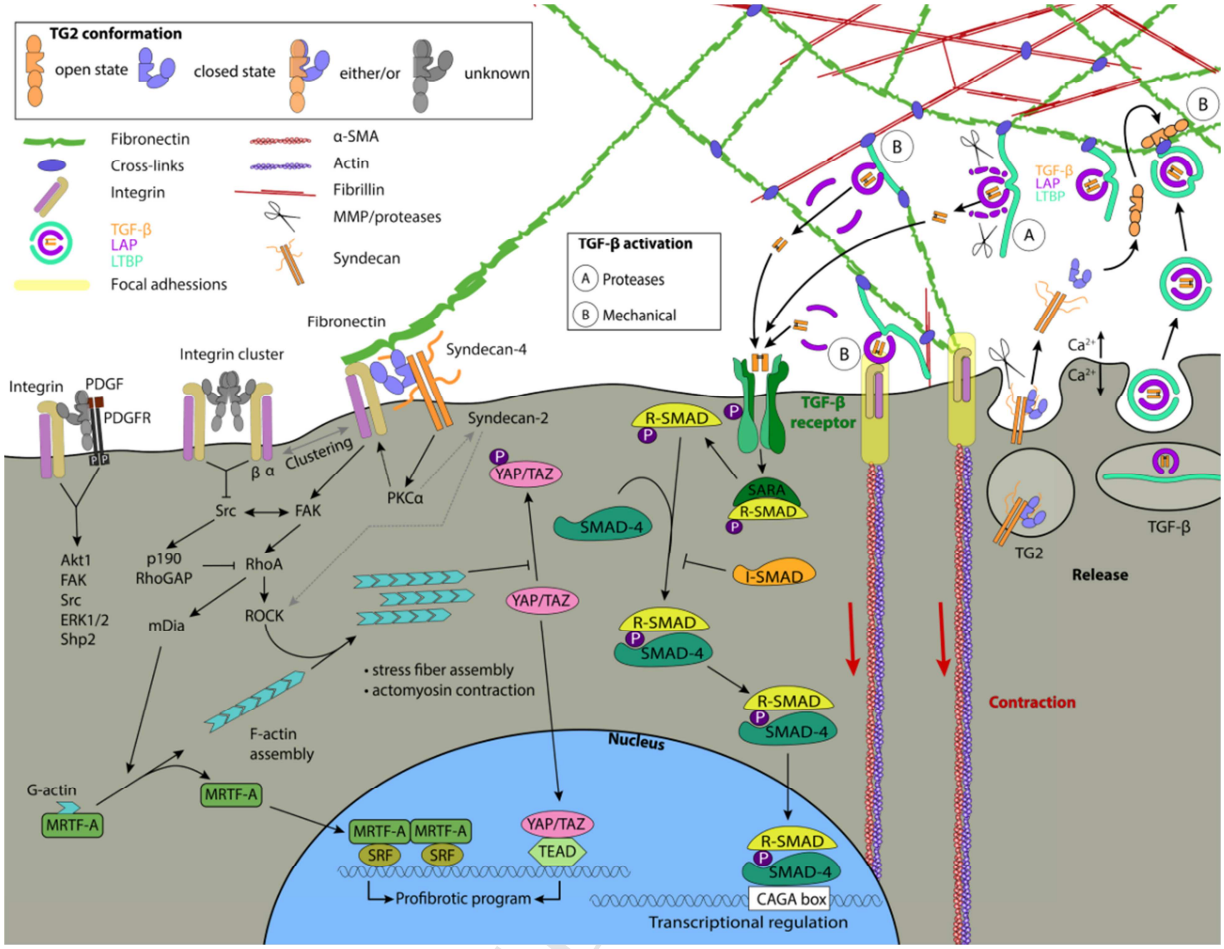

Figure 3:

Key pro-fibrotic roles of TG2 at the cell membrane and in the ECM.

The molecular sketches do not represent molecular dimensions nor potential conformational states. Unconventional cellular release and reuptake of TG2 are not depicted in this scheme. Figures are adapted and modified with permission from Belkin et al. [7] and Robertson et al. [75].

<u>Membrane associated functions of TG2 (lower left section of figure):</u>

TG2-integrin clusters, TG2-FN-Integrin and TG2-FN-integrin-syndecan-4 complexes are known to activate intracellular Src-FAK and inhibit p190RhoGAP through Src, with both pathways leading to RhoA-ROCK activation [7,8,19,29,41]. RhoA/ROCK pathway activation induces the assembly of cytoplasmic monomeric G-actin into F-actin fibers and the formation of stress fibers. Changes in actin dynamics are monitored by myocardin-related transcription factor A (MRTF-A), which binds to cytoplasmic G-actin, but dissociates from F-actin. Upon actin fiber assembly MRTF-A thus uncouples from G-actin and the released MRTF-A enters the nucleus. Alternatively, Rho-signaling and cytoskeletal stress drive the downregulation of YAP/TAZ phosphorylation and the subsequent nuclear translocation and activation of YAP/TAZ. Nuclear MRTF-A and YAP/TAZ both act as transcription cofactors that promote profibrotic gene expression [46,47]. RGD independent cell adhesion to Fn depends on Fn-TG2-syndecan-4

interaction and is mediated via cytoplasmic α5β1 integrin and syndecan-2 activation by Syndecan-4 activated PKCα [29]. Membrane-associated TG2 interacts with growth factor receptors (such as for PDGF, VEGF, FGF and EGF) and stimulates cell survival signaling pathways (e.g. FAK, Src, Akt, etc.) by promoting GF-receptor binding and activation [7,21].

Storage of latent TGF-β1 in the ECM and mechanoactivation of TGF-β1 (right section of figure):
TGF-β1 is secreted by cells in complex with its latency associated propeptide (LAP), which associates with latent TGF-β1 binding protein (LTBP-1) [76]. LTBP-1 facilitates secretion of TGF-β1 [77] and TG2 cross-links LTBP-1 to Fn and fibrillin ECM fibers [16,34]. As such, a matrix reservoir of inactive TGF-β1 is formed which is bound to the LTBP-1 and ECM and therefore unable to bind with its high affinity cell membrane receptor to activate the downstream SMAD-mediated signaling cascade [34,74]. Latent TGF-β1 can be activated through its release from the ECM-bound TGFβ-LAP-LTBP-1 complex by proteolysis or integrin mediated cellular contraction [33–35]. The N- and C-terminal matrix binding sequences of LTBP-1 [78] and integrin binding RGD sequence of LAP [79] are critical for the activation of TGF-β1. Cell membrane associated integrins bind to the RGD sequence on LAP and can expose the ECM bound LTBP-1 to cellular contractile forces. This is especially effective when the LTBP-1 is bound to a stiff, deformation resistant ECM that counters cell-applied forces [17,33,35,36]. As such, TGF-β1 activation is thought to be facilitated by TG2 since TG2 cross-links LTBP to the ECM and can stiffen a compliant matrix via ECM cross-linking [37]. For a detailed review on TGF-β-LTBP interactions we refer the reader to a recent comprehensive review published by Rifkin et al. [74].

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   Using biochemical and biophysical approaches, this study identified the TG2 residues K30, R116 and H134 in the N-terminal domain as critical for high affinity interaction of TG2 with Fn. Interestingly, whether TG2 adopted an open or closed state conformation did not influence Fn-binding affinity.

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The authors used a pulmonary fibrosis cell culture (IPF fibroblasts) and an in vivo murine pulmonary fibrosis model to examine the expression of TG2 and LOX and the effects of their inhibition on fibroblast growth and ECM turnover. IPF fibroblasts strongly expressed TG2 and TG2 inhibition decreased fibroblast adhesion and proliferation and increased ECM turnover in vitro. TG2 inhibition decreased the total collagen content in the lungs of treated compared to control mice.

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AUTHOR DECLARATION TEMPLATE

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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14.04.2019 Mario Benn

14.04.2019 Willi Weber

16.04.2019 Viola Vogel